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The Arabidopsis TCH4 Xyloglucan Endotransglycosylase¹

Substrate Specificity, pH Optimum, and Cold Tolerance

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Xyloglucan endotransglycosylases (XETs) modify a major component of the plant cell wall and therefore may play critical roles in generating tissue properties and influencing morphogenesis. An XET-related gene family exists in *Arabidopsis thaliana*, the members of which show differential regulation of expression. *TCH4* expression is rapidly regulated by mechanical stimuli, temperature shifts, light, and hormones. As a first step in determining whether Arabidopsis XET-related proteins have distinct properties, we produced recombinant TCH4 protein in bacteria and determined its enzymatic characteristics. TCH4 specifically transglycosylates only xyloglucan. The enzyme prefers to transfer a portion of a donor polymer onto another xyloglucan polymer (acceptor); TCH4 will also utilize xyloglucan-derived oligosaccharides as acceptors but discriminates between differentially fucosylated oligosaccharides. TCH4 is most active at pH 6.0 to 6.5 and is surprisingly cold-tolerant with an optimum of 12 to 18°C. TCH4 activity is enhanced by urea and bovine serum albumin, but not cations, reducing agents, or carboxymethylcellulose. These studies indicate that TCH4 is specific for xyloglucan, but that the molecular mass and the fucosyl content of the substrates influence enzymatic reaction rates. TCH4 is unlikely to play a role in acid-induced wall loosening but may function in cold acclimation or cold-tolerant growth.

Plant cell growth is a highly regulated process that is likely controlled in part by the activity of cell wall-modifying enzymes. The cell wall is a dynamic network of cellulose, hemicellulose, pectin, and proteins (for review, see Carpita and Gibeau, 1993; McCann and Roberts, 1994). In dicotyledons the major hemicellulose in primary cell walls is xyloglucan. Xyloglucan consists of a 4-linked β -D-glucan backbone with approximately 75% of the backbone glucosyl residues substituted at O-6 with an α -D-xylosyl residue (Bauer et al., 1973; Hayashi, 1989; Zablackis et al., 1995; for review, see Fry, 1989a). Some xylosyl residues are

substituted at O-2 with β -D-galactopyranosyl residues, and some of these β -D-galactopyranosyl residues are themselves substituted with α -L-fucopyranosyl residues. Xyloglucans hydrogen bond to the cellulose microfibrils in the wall (Valent and Albersheim, 1974), thereby probably cross-linking them (Hayashi et al., 1987, 1994a, 1994b; Hayashi 1989; Fry, 1989b). Modification of these cellulose-xyloglucan cross-links is most likely a key step in controlling cell extensibility. Recently, the enzyme XET has been identified in and purified from plants (Smith and Fry, 1991; Farkas et al., 1992; Fry et al., 1992; Nishitani and Tominaga, 1992; Fanutti et al., 1993). XETs cleave xyloglucan polymers internally and transfer the newly generated ends to other xyloglucan molecules. Thus, XETs may be responsible for incorporation of new xyloglucan into the growing cell wall (Edelmann and Fry, 1992; Nishitani and Tominaga, 1992; Talbott and Ray, 1992; Xu et al., 1996) or rearranging xyloglucan polymers during cell wall loosening, leading to cell expansion (Fry, 1989b; Smith and Fry, 1991; Fry et al., 1992).

An extensive gene family encoding XET-related proteins has been identified in *Arabidopsis thaliana*. This family of proteins includes TCH4, Meri-5, EXT, and at least five XET-related proteins (XTRs), which share 37 to 84% identity (Medford et al., 1991; Okazawa et al., 1993; Xu et al., 1995; Xu et al., 1996). A recombinant TCH4 protein produced in *Escherichia coli* has been shown to be an XET because it is able to catalyze the incorporation of a xyloglucan-derived ³H-oligosaccharide into polymeric xyloglucan (Xu et al., 1995).

The expression of *TCH4* is up-regulated by touch, darkness, heat shock, cold shock, increased external calcium, auxin, and brassinosteroids, with transcript levels reaching a maximum between 10 min and 2 h (Braam and Davis, 1990; Braam, 1992; Polisensky and Braam, 1996; Xu et al., 1996). This suggests that TCH4 may play a role in plant

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Abbreviations: XET, xyloglucan endotransglycosylase; XLLGol, xyloglucan-derived nonasaccharide, with one xylosyl-substituted Glc, two galactosyl-xylosyl-substituted Glc's, and an unsubstituted glucitol; XXFGol, xyloglucan-derived nonasaccharide, with two xylosyl-substituted Glc's, one fucosyl-galactosyl-xylosyl-substituted Glc, and an unsubstituted glucitol.

responses to environmental and hormonal stimuli through modification of the cell wall. For example, plants that are repeatedly touched generally grow shorter and stockier and may increase in strength and flexibility (for review, see Biddington, 1986; Jaffe and Forbes, 1993; Mitchell and Myers, 1995); these changes likely involve cell wall modifications. Auxin and brassinosteroid enhance growth; increased *TCH4* expression may be an important step in hormone-induced alterations for cell expansion. The expression of a *TCH4-GUS* reporter gene in transgenic Arabidopsis is strong in growing regions, such as young expanding leaves, developing trichomes, emerging lateral root primordia, and the elongating hypocotyls of seedlings grown in low light (Xu et al., 1995). In addition, total XET activity from plants shows positive correlations with the growth rate in many tissues (Potter and Fry, 1993, 1994; Pritchard et al., 1993).

Expression of the Arabidopsis XET-related genes is differentially regulated by environmental and hormonal stimuli, and the magnitude and kinetics of regulation are distinct for the different genes (Xu et al., 1996). It is likely that the members of the gene family are spatially and temporally regulated during development, as well (Xu et al., 1996). It remains to be determined whether all of the XET-related proteins have XET activity, but based on their strong sequence conservation, it is likely that they have transglycosylase activity. However, the extent of amino acid sequence divergence of some of the members indicates that the different XET-related proteins may have distinct characteristics, such as substrate preference or pH optima. Differential regulation of expression of genes encoding cell wall enzymes with specific and diverse enzymatic activities may contribute to the precise composition and arrangement of polymers in the cell wall. Here, we have focused on the XET encoded by *TCH4* and have carried out an extensive characterization of the enzymatic properties. *TCH4* shows an unusual temperature dependence and unexpected preference for a nonfucosylated xyloglucan oligosaccharide. The identification of these properties is the first step in understanding possible functional distinctions between the XET-related proteins in Arabidopsis.

MATERIALS AND METHODS

Recombinant TCH4 Production and Purification

TCH4 was produced in *Escherichia coli* and purified as previously described (Xu et al., 1995). Briefly, the DNA region encoding the putative mature *TCH4* polypeptide (lacking the putative signal sequence) was amplified by the PCR, cloned into pET21 (Novagen, Madison, WI), adding a C-terminal six-residue His tag, and sequenced. The DH5 α cells harboring the expression vector were infected with λ bacteriophage CE6. *TCH4* was found primarily in the insoluble fraction of the bacterial lysate and was solubilized in 8 M urea. The protein was loaded onto a 1-mL nickel-charged column (Novagen) and subjected to a reverse urea gradient (8 to 1 M, 2.5-mL washes), eluted with imidazole/1 M urea, and dialyzed against 20% glycerol, 0.5 M

NaCl, and 0.04 M Tris-HCl, pH 7.4, with two changes over 36 h at 4°C. Protein concentration was estimated by the Micro-BCA kit (Pierce) using BSA as a standard.

SDS-Polyacrylamide Analysis of Recombinant TCH4

Bacteriophage CE6 (Novagen) was used to induce target gene expression in control cells (pET21 vector only) or the experimental cells (*TCH4* in pET21). Sixty microliters of bacterial culture was centrifuged, resuspended in 1 \times Laemmli's buffer (60 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT, 0.01% bromophenol blue), boiled for 3 min, and separated by SDS-10% PAGE. Twelve microliters of column-purified *TCH4* (approximately 12 μ g) was also subjected to electrophoresis to monitor *TCH4* purification. Proteins were visualized by staining with Coomassie brilliant blue.

Oligosaccharides and Polysaccharides

[1-³H]XLLGol (18 or 20 MBq/ μ mol), unlabeled XLLGol, [1-³H]XXFGol (22.4 MBq/ μ mol), and tamarind xyloglucan were prepared as previously described (Hetherington and Fry, 1993). Fucosylated xyloglucan from pea (*Pisum sativum* L.) stems was the generous gift of Dr. Takahisa Hayashi (Wood Research Institute, Kyoto University, Japan). Other polysaccharides were purchased from Sigma and BDH (Poole, Dorset, UK).

Standard XET Assay

All of the experiments used the following basic methods except where otherwise stated. Reaction mixtures (final volume 20 to 25 μ L) contained 1 mg of tamarind xyloglucan/mL, 3.8 to 4.9 kBq of [³H]XLLGol (5–34 μ M), citrate or succinate buffer, pH 6.0 (200–360 mM), and 0.35 to 1.7 μ g of *TCH4*. For the temperature-optimum experiment, 5 μ L of crude *E. coli* extract (soluble fraction) was used, and the succinate buffers were adjusted to pH 6.0 at each temperature that was used in the assay. For the determination of preference for fucosylated substrates, each xyloglucan was used at 0.04 mg/mL. Reactions were incubated for 4 to 8 h and stopped by the addition of 70 μ L of 30% (v/v) formic acid. The reaction mixtures were dried onto Whatman 3MM paper and radioactive polymer was quantified as previously described (Fry et al., 1992).

Assay of Endotransglycosylation of Polysaccharides Other Than Xyloglucan

Other polysaccharides were tested as the substrates for *TCH4* by paper chromatography as previously described (Fry et al., 1992).

Extraction of XET Activity from Arabidopsis

Fresh shoot tissue from 11 26-d-old *Arabidopsis thaliana* plants, ecotype RLD, was extracted by grinding in 1.5 mL

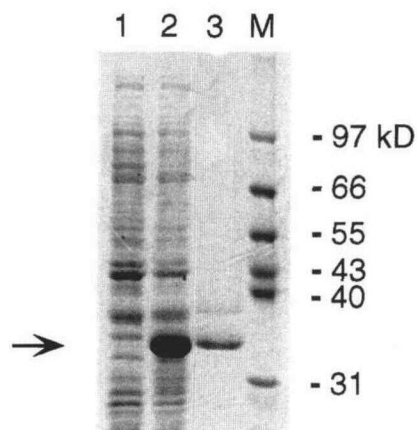


Figure 1. Purification of recombinant TCH4 from *E. coli*. Samples were separated on an SDS-10% polyacrylamide gel and stained with Coomassie blue. Lane 1, Total lysate from cells transformed with pET21 vector only; lane 2, total lysate from cells transformed with TCH4 in pET21; and lane 3, TCH4 purified from a nickel column. The arrow indicates TCH4. A less abundant, slightly larger band of unknown identity coeluted from the column. Lane M, Molecular mass standards (in kD at right).

of ice-cold 0.5 M NaCl in a mortar and pestle with acid-washed sand. The mixture was centrifuged for 5 min at 4°C, and 5 μ L of the supernatant was used in the XET assays.

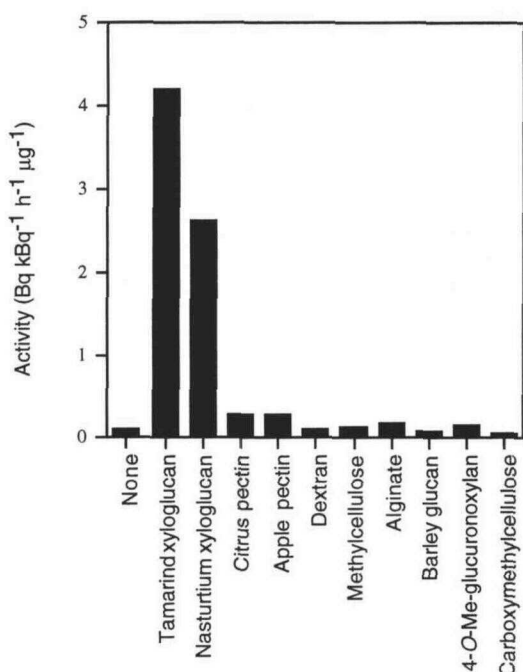


Figure 2. Donor substrate specificity of TCH4. Each polysaccharide was tested at 1 mg/mL as a possible donor substrate for TCH4 in the presence of [³H]XLLGol as the acceptor. ³H-Labeled polysaccharides formed by the activity of TCH4 were separated from [³H]XLLGol by paper chromatography. Activity is reported as Bq of ³H-labeled polysaccharides formed per kBq of [³H]XLLGol per hour per μ g of TCH4. 4-O-Me-glucuronoxylan, 4-O-Methyl-glucuronoxylan.

RESULTS AND DISCUSSION

Production of Recombinant TCH4

To generate a mature TCH4 protein, we amplified a TCH4 gene fragment from a TCH4 cDNA using the PCR. The fragment extended from Asn-22 to Ala-284, eliminating the putative N-terminal signal sequence and the stop codon (Xu et al., 1995). The gene fragment was cloned into the pET21 (Novagen) *E. coli* expression vector to add six His residues to the C terminus as a tag to simplify purification of the TCH4 protein. The recombinant vector was transformed into BL21(DE3)pLysS expression cells (Novagen). The expression of TCH4 from this construct is under the control of the isopropyl β -D-thiogalactopyranoside-inducible T7 *lac* promoter, which is designed to have very low constitutive expression. However, the trace amount of constitutive expression is most likely detrimental to bacteria, because we could not recover cells capable of producing TCH4. To avoid basal expression of the TCH4 gene, we transferred the TCH4 expression construct into DH5 α cells, which lack the T7 RNA polymerase-encoding gene. To induce TCH4 gene expression we infected the cells with the CE6 bacteriophage that bears the T7 RNA polymerase gene (Novagen). Under these conditions the TCH4 protein was readily detectable on SDS-PAGE (Fig. 1, lane 2) when com-

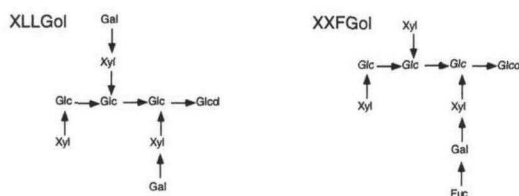
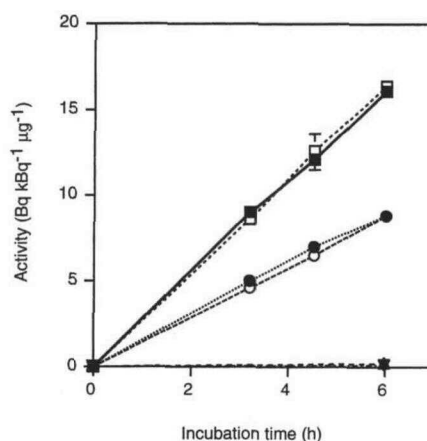


Figure 3. Dependence of TCH4 activity on the fucosylation state of substrates. The transfer of an unlabeled polysaccharide (0.04 mg/mL) to a ³H-labeled oligosaccharide was measured. Activity is reported as Bq ³H-polysaccharide formed per kBq of ³H-oligosaccharide per microgram of TCH4 protein. Each point represents the mean \pm SD for triplicate determinations. ■, XLLGol, nonfucosylated xyloglucan; ●, XXFGol, nonfucosylated xyloglucan; □, XLLGol, fucosylated xyloglucan; ○, XXFGol, fucosylated xyloglucan; ▲, XLLGol, no xyloglucan; and ▼, XXFGol, no xyloglucan.

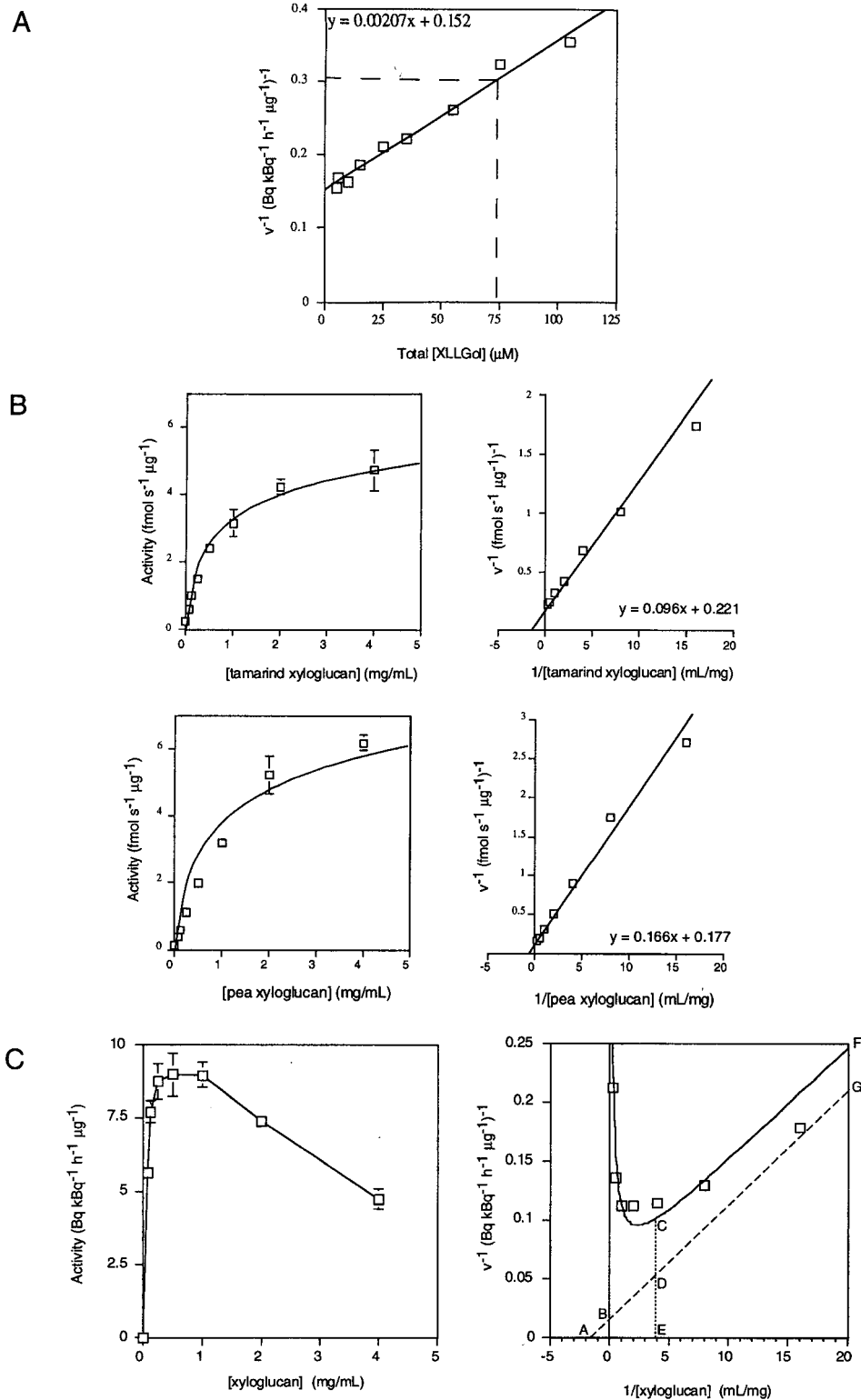


Figure 4. A, Determination of K_m for XLLGol. Unlabeled XLLGol was added in increasing concentrations to the reaction with a fixed concentration of $[^3\text{H}]\text{XLLGol}$ of $4.6 \mu\text{M}$. The K_m was calculated from the concentration of unlabeled XLLGol that decreased the rate of ^3H incorporation 2-fold. B, Determination of K_m and V_{\max} for tamarind (nonfucosylated) and pea (fucosylated) xyloglucan. Xyloglucan concentration was varied from 0.06 to 4 mg/mL, while holding constant the

(Legend continues on facing page.)

pared with control cells (Fig. 1, lane 1). The TCH4 protein accumulated as insoluble material in *E. coli*. We solubilized the protein with 8 M urea, then purified TCH4 on a nickel-charged column with a reverse urea gradient to 1 M urea to remove the urea slowly and refold the protein. Dialysis against urea-free buffer removed the last traces of urea. Significant purification was obtained with this procedure (Fig. 1, lane 3).

Substrate Specificity

To test the substrate specificity of TCH4, we examined its ability to catalyze the transfer of various polysaccharides (donor substrates) onto a xyloglucan-derived ^3H -oligosaccharide (acceptor substrate). TCH4 was specific for polymeric xyloglucan (Fig. 2). No other tested polysaccharide, including other β -glucans such as carboxymethylcellulose and barley glucan, was an efficient substrate for TCH4 (Fig. 2). The slight activity seen with pectins may have been due to traces of xyloglucan present in the commercial preparations of the pectin that we used. TCH4 was more active against tamarind xyloglucan than nasturtium xyloglucan, perhaps due to subtle differences in xyloglucan side-chain composition or organization. These findings are consistent with previous reports of the specificity of XET activity from pea stems for xyloglucan (Fry et al., 1992).

In some tissues the β -D-galactosyl- α -D-xylosyl residues are L-fucosylated. Levy and colleagues (Levy et al., 1991, 1997) have provided evidence that the trisaccharide side chain (Fuc-Gal-Xyl) forces the xyloglucan backbone into a more rigid, straight conformation that may make the xyloglucan in this region more accessible for cellulose binding. The *Arabidopsis mur1* mutant, which substitutes L-Gal for L-Fuc, has stems with reduced tensile strength (Reiter et al., 1993; Zablackis et al., 1996). In *Zinnia* sp. xyloglucan is fucosylated in tissues that require high tensile strength, such as the epidermis and vascular bundles, whereas xyloglucan is not fucosylated in mesophyll cells, where intercellular connections are most likely weaker to allow air spaces to form (McCann and Roberts, 1994). Certain seeds, such as those of nasturtium and tamarind, have no Fuc in their storage xyloglucans, and XETs have been identified that preferentially transglycosylate nonfucosylated xyloglucan (Fanutti et al., 1993; Rose et al., 1996). In a preliminary experiment to determine whether the presence of Fuc in the donor substrate influences TCH4 activity, we assayed TCH4 activity against pea stem xyloglucan, which is fucosylated, and tamarind seed xyloglucan, which is not. Each was tested at a concentration of 1 mg/mL, with 4.2 μM [^3H]XLLGol as an acceptor. TCH4 was equally active against each xyloglucan polymer tested (Fig. 3). However,

when we assayed TCH4 activity using differentially fucosylated acceptors, [^3H]XLLGol (containing two galactosyl substitutions) and [^3H]XXFGol (containing one galactosyl-fucosyl substitution), we found a difference in activity. Activity was reduced by approximately 50% using the same concentration of [^3H]XXFGol (Fig. 3).

Determination of K_m for XLLGol as an Acceptor and Xyloglucan as a Donor

The apparent sensitivity of TCH4 to fucosylation and/or galactosylation of the acceptor, but not the donor, substrate indicated that more precise kinetic data were required so that we could assay the enzyme under conditions in which either the xyloglucan (donor) concentration or the oligosaccharide (acceptor) concentration was limited in the reaction, such that either $E + DX \rightarrow ED + X$ or $ED + A \rightarrow E + DA$ would be rate-limiting for ^3H incorporation, where E is the enzyme, A is the acceptor substrate, DX is the donor substrate, D is the portion of the donor (including nonreducing terminus) transferred to the acceptor, and X is the remainder (including reducing terminus) of the donor.

We first determined the K_m for XLLGol as acceptor substrate by adding increasing concentrations of unlabeled XLLGol to the [^3H]XLLGol + xyloglucan + TCH4 reaction and determining the concentration at which the competing XLLGol halved the reaction rate (Fig. 4A). This concentration was determined to be 73 μM . When assayed with an excess of XLLGol (250 μM) as acceptor substrate, TCH4 had a higher affinity for nonfucosylated xyloglucan as the donor substrate, with a K_m of 0.62 mg/mL, than for fucosylated xyloglucan, with a K_m of 1.8 mg/mL (Fig. 4B). However, the V_{\max} for pea xyloglucan, 9.3 fmol $\text{s}^{-1} \mu\text{g}^{-1}$, was greater than the V_{\max} for tamarind xyloglucan, 5.5 fmol $\text{s}^{-1} \mu\text{g}^{-1}$ (Fig. 4B). Turnover numbers for TCH4, assayed with saturating concentrations of XLLGol and xyloglucan, were estimated to be $3.1 \times 10^{-4} \text{ s}^{-1}$ (pea xyloglucan) and $1.8 \times 10^{-4} \text{ s}^{-1}$ (tamarind xyloglucan). Although these turnover numbers are low, the reaction rates were constant for at least 11 h, indicating that the TCH4 protein was acting as a catalyst, not a substrate. At present we do not know whether these low values for turnover numbers are characteristic of native plant TCH4 or a consequence of the purification of a recombinant TCH4 from *E. coli*, including (a) the addition of amino acids to the termini of TCH4 from the expression vector; (b) the absence of posttranslational modifications that occur in the plant, such as glycosylation; and/or (c) incomplete renaturation during removal of the urea. Because the rate of product formation is constant for 11 h (Xu et al., 1995), we believe that the low turnover numbers indicate the presence of inactive protein. There-

(Continued from facing page.) concentration of [^3H]XLLGol (250 μM) and other reaction constituents. Activity was determined as described in "Materials and Methods" and is reported as fmol of ^3H -labeled polysaccharide formed per second per microgram of TCH4. Each point represents the mean \pm SD for triplicate determinations. C, Estimation of K_m of tamarind xyloglucan for the TCH4 acceptor-binding site by its ability to compete with [^3H]XLLGol (8 μM). Left, Increasing concentrations of xyloglucan (x axis) and low concentrations of XLLGol will favor polysaccharide-to-polysaccharide transglycosylation over polysaccharide-to-oligosaccharide transglycosylation, thus reducing the reaction rate (y axis) with the ^3H -oligosaccharide. Right, Lineweaver-Burk plot of the data in the left panel. The curve is drawn from an equation that accounts for the xyloglucan polymer acting as a competitive inhibitor (for details, see "Appendix").

fore, it is likely that the activities detected reflect properties of a minority of recombinant TCH4 protein that is properly folded and fully active.

Determination of K_m for Xyloglucan as Acceptor

TCH4 appears to be capable of catalyzing transglycosylation between two xyloglucan polymers, since polymeric xyloglucan could compete with [^3H]XLLGol in the reaction. When [^3H]XLLGol was used at 8 μM (a concentration well below its K_m as acceptor substrate), and xyloglucan was used at up to 4 mg/mL (concentrations above its K_m as donor substrate), we observed a decrease in the rate of ^3H incorporation at high xyloglucan concentrations (Fig. 4C, left). A Lineweaver-Burk plot generated from these data is clearly not linear, consistent with xyloglucan binding to the enzyme's acceptor binding site (Fig. 4C, right). Xyloglucan is a competitive inhibitor for this site, because the Lineweaver-Burk plots in Figure 4B (in which a great excess of [^3H]XLLGol was used) are linear. We were able to make an estimate of the K_m of TCH4 for xyloglucan as the acceptor substrate by considering xyloglucan (the donor) to be a competitive inhibitor of [^3H]XLLGol utilization as the acceptor substrate. For a detailed description of the analysis, see "Appendix."

Briefly, we derived an equation (Eq. 7; "Appendix") describing the relationship between v (the reaction velocity), $V_{\max(d)}$ (the theoretical maximum velocity that would have been expected at infinite donor concentration and an arbitrarily chosen [^3H]XLLGol concentration if xyloglucan had not acted as an acceptor substrate), xyloglucan concentration, and K_i (the dissociation constant of the enzyme-inhibitor complex, i.e. the K_m of TCH4 for xyloglucan as the acceptor substrate). We then used the Marquardt-Levenberg algorithm to generate a value for K_i and $V_{\max(d)}$ that would best fit the experimental data points. The curve using these values is shown in Figure 4C, right. We then generated the hypothetical Lineweaver-Burk linear plot that would have been expected with this value of $V_{\max(d)}$ if xyloglucan had not competed for the acceptor site (Fig. 4C, right, line AG). We calculated that under the conditions of our experiment ([^3H]XLLGol = 8 μM), and when $[I] = K_i$, the effective enzyme concentration is 52.6% of what it would have been if xyloglucan had not acted as a competitive inhibitor, and we estimated the K_m of TCH4 for xyloglucan as the acceptor substrate ($=K_i$) from the point at which the hypothetical $1/v = 52.6\%$ of the observed $1/v$ (Fig. 4C, right, DE = 52.6% of CE). Thus, we found the K_m of TCH4 for xyloglucan as the acceptor substrate to be approximately 0.26 mg/mL, which equals approximately 0.3 μM (to one significant figure).

We assume that tamarind xyloglucan has an unbranched β -glucan backbone, and that the only acceptor site in the xyloglucan molecule is the nonreducing terminus of that backbone (Nishitani and Tominaga, 1992). Two observations indicate that the number of nonreducing termini did not appreciably change during the reaction: (a) in the absence of oligosaccharides, TCH4 did not affect the viscosity of solutions of tamarind xyloglucan, suggesting that endohydrolysis did not significantly compete with transgly-

cosylation; and (b) the TCH4 reaction rates were approximately constant for about 11 h (Xu et al., 1995; data not shown). Thus, the most relevant acceptor concentration is the molarity of the xyloglucan polymer (rather than mg/mL). The data show that the acceptor-substrate binding site of the TCH4 protein has a much higher affinity for xyloglucan (K_m approximately 0.3 μM) than for XLLGol (K_m approximately 73 μM).

Takeda et al. (1996) detected, in extracts of cultured poplar cells, an XET activity in which the preferred acceptor substrate was the Glc₈-based tetradecasaccharide, XXXGXXXGol. The corresponding Glc₄- and Glc₁₂-based oligosaccharides were less effective, and the Glc₁₆-based octacosasaccharide (XXXGXXXGXXXGXXXGol; M_r approximately 4,200) did not act as an acceptor at all. Pea xyloglucan (M_r approximately 50,000) also failed to act as an acceptor substrate, as shown by the linearity of the Lineweaver-Burk plot for 1/incorporate rate (of ^3H from 5 μM [^3H]XXXGol) versus 1/xyloglucan concentration (tested at up to 100 μM) (contrast figure 4 of Takeda et al. with our Fig. 4C, right). Two XETs thus differ radically in their acceptor substrate specificity: the TCH4 enzyme prefers a polymeric acceptor, whereas the poplar XET prefers a tetradecasaccharide. This supports the hypothesis that different XETs serve different roles in modification of the cell wall.

In vivo concentrations of xyloglucan in the cell wall matrix are approximately 100 mg/mL (Fry, 1989a), or about 500 μM , assuming a typical M_r for primary wall xyloglucan of 200,000 (Thompson and Fry, 1997). Thus, the xyloglucan concentration in the cell wall matrix greatly exceeds the K_m values of both the donor- and acceptor-binding sites of TCH4 as assayed in vitro. However, we cannot assume from this that in vivo TCH4 will be catalyzing transglycosylation at a rate close to the V_{\max} , because we do not know whether in the wall matrix the xyloglucan (and especially its nonreducing terminus) is readily accessible to the enzyme. In addition, it is not known whether the nonreducing terminus of xyloglucan in vivo normally bears the α -D-Xyl residue that is required for acceptor function (Lorences and Fry, 1993). Cell walls often contain α -D-xylosidases, which can remove this residue. Only direct measurements of the metabolism of xyloglucan in vivo will provide information on the rate of transglycosylase action in the walls of living cells.

pH Optimum

To determine the optimal pH for TCH4, we assayed activity in buffers ranging from pH 2.5 to 8.0, using each buffer system in its effective range. The activity of TCH4 was highest between pH 6.0 and 6.5 (Fig. 5). TCH4 activity must be influenced slightly by the composition of the buffer, as can be seen from a pH optimum of 6.0 in succinate buffer and 6.5 in citrate buffer (Fig. 5). This pH was higher than the 5.5 reported for XET activity from pea stems (Fry et al., 1992). It is possible that different XETs have distinct pH optima; transient changes in cell wall pH could therefore recruit specific XET isozyme activity in processes such as auxin-induced growth. XETs with rela-

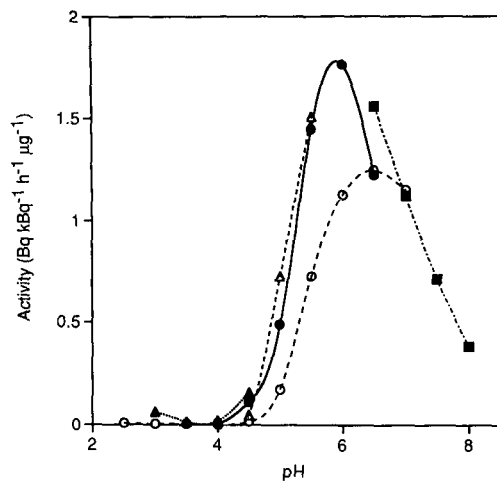


Figure 5. The pH dependence of TCH4 activity on [^3H]XLLGol and xyloglucan. The buffers that were used were (all 190 mM, with Na^+ as counterion): \blacktriangle , formate; \triangle , acetate; \bullet , succinate; \blacksquare , phosphate; and \circ , citrate. Activity is reported as Bq of ^3H -polysaccharide formed per kBq of [^3H]XLLGol per hour per μg of TCH4. Each point represents the mean of triplicate determinations. The results were nearly identical in an independent experiment.

tively low pH optima may act synergistically with expansins, which are known to promote acid-induced cell wall loosening and have a pH optimum of 3.5 to 4.5 (McQueen-Mason et al., 1992; McQueen-Mason, 1995), whereas other XETs, such as TCH4, may be involved in different processes, such as assembling nascent cell walls or reinforcing existing and expanding walls.

Temperature Optimum

The expression of *TCH4* is up-regulated by heat shock, cold shock, and during cold acclimation (Braam, 1992; Polisensky and Braam, 1996; D.H. Polisensky and J. Braam, unpublished results). If *TCH4* functions to modify the cell wall at these temperature extremes, it may be expected that *TCH4* would retain much of its activity at these temperatures. We therefore tested the activity of *TCH4* at different temperatures, ranging from -5 to 44°C . For this experiment, we used the soluble fraction of an extract from the *E. coli* producing *TCH4* to eliminate any influence of the in vitro refolding of *TCH4* on the protein's thermostability. It is interesting that *TCH4* exhibited its highest activity at 12 to 18°C (Fig. 6), which is significantly lower than the recommended temperature of 25°C for the growth of *Arabidopsis* (Arabidopsis Biological Resource Center Seed and DNA Stock List, 1995).

To assess whether this is a property common to the majority of *Arabidopsis* XETs, we determined the temperature profile of total XET activity from a crude extract of *Arabidopsis* shoot tissue. Total XET activity was highest at about 30°C (Fig. 6). Therefore, the cold preference of *TCH4* appears to be unusual, at least compared with the bulk of XET activity present in young *Arabidopsis* shoot tissue. The recombinant *TCH4* protein was also more heat-sensitive than the total XETs assayed, with undetectable

activity at 44°C . Thus, the *TCH4* protein appears to be more active at lower temperatures (at and below 18°C) and less active at higher temperatures (above 26°C); however, *TCH4* is not simply denatured at temperatures higher than 18°C , because the rate of product formation is constant for at least 11 h at 25°C (Xu et al., 1995). *TCH4* is the only XET-related gene known to be up-regulated in expression by cold shock; three other XET-related genes are down-regulated by cold shock (Xu et al., 1996). Thus, it is possible that *TCH4* is a unique *Arabidopsis* XET-related that is up-regulated in expression by cold, and that the *TCH4* protein is particularly suited to functioning at low temperatures.

Activity in the Presence of Cations, Reducing Agents, Urea, Albumin, and Carboxymethylcellulose

To examine further the properties of the *TCH4* enzyme, we tested its activity in the presence of various additives. Although the expression of *TCH4* is up-regulated by increased cytosolic calcium (Braam, 1992), *TCH4* activity was not enhanced by calcium or other divalent cations (Table I). *TCH4* has four Cys residues near the C terminus that may form disulfide bridges (Xu et al., 1995). Recombinant *TCH4* activity was not significantly altered by the addition of reducing agents (Table I). Activity was enhanced by the addition of urea at various concentrations (Table I). There are two possible explanations for this. First, it is possible that *TCH4*, having been refolded after denaturation, is not entirely and/or correctly folded, and thus a partial denaturation by low concentrations of urea may allow a more correct conformation of the protein. Second, the chaotropic effects of urea may weaken hydrogen bonding between

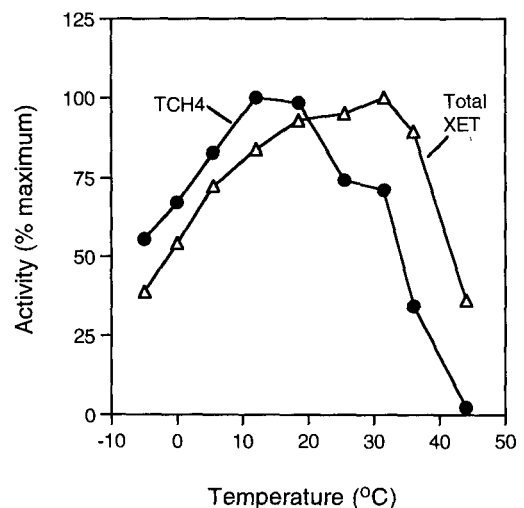


Figure 6. Temperature profile of *TCH4* and total plant XET activity. *TCH4* activity in the soluble fraction of an *E. coli* extract (\bullet) or total XET activity in a crude extract from 26-d-old *Arabidopsis* plants (\triangle) was assayed at the temperatures shown. Activity was measured as Bq of ^3H -polysaccharide formed per kBq of [^3H]XLLGol in a 2-h reaction (plant XET activity) or in a 3-h reaction (*E. coli* extract) and then normalized to show percent of maximum activity. Each point represents the mean for duplicate determinations.

Table 1. Influence of various agents on the activity of TCH4

TCH4 activity on xyloglucan and [^3H]XLLGol was measured in the presence of the additive listed. Activities are expressed as a percentage of activity under control conditions, i.e. without other additions. The results are means \pm SD for three to six determinations.

Additive	Concentration	XET Activity
		% of control
None		100 \pm 6
CaCl_2	10 mM	111 \pm 30
MgCl_2	10 mM	124 \pm 21
MnCl_2	10 mM	114 \pm 7
KCl	100 mM	94 \pm 12
2-Mercaptoethanol	10 mM	111 \pm 9
DTT	10 mM	72 \pm 14
Ascorbate	10 mM	114 \pm 8
Glutathione	10 mM	116 \pm 15
Spermidine	1 mM	101 \pm 12
Spermine	1 mM	111 \pm 8
Glycerol	2.5%	122 \pm 13
BSA	0.01%	346 \pm 15
BSA	0.1%	271 \pm 121
BSA	0.2%	271 \pm 20
Urea	1 M	169 \pm 58
Urea	1.5 M	237 \pm 15
Carboxymethylcellulose	0.2%	120 \pm 18

xyloglucan polymers to improve the accessibility of the substrate. It has been observed that low concentrations of urea enhance the effects of expansins, presumably by decreasing the extent of hydrogen bonding between cellulose and xyloglucan (McQueen-Mason and Cosgrove, 1994; McQueen-Mason, 1995). TCH4 activity was also enhanced by the addition of BSA at various concentrations (Table I). Most likely, the albumin stabilizes TCH4 conformation by increasing the protein concentration of the solution. The activity was not significantly altered by spermidine, spermine, or glycerol (Table I). Finally, because xyloglucan is found hydrogen-bonded to cellulose microfibrils in the cell wall, we tested the activity of TCH4 in the presence of carboxymethylcellulose, a soluble cellulose derivative. TCH4 activity was not altered by the addition of carboxymethylcellulose (Table I). This lack of effect could be because TCH4 activity is not influenced by the cellulose binding of xyloglucan, or because the interaction of xyloglucan and carboxymethylcellulose *in vitro* is very different from that of xyloglucan and crystalline cellulose microfibrils in the cell wall.

CONCLUSION

In conclusion, recombinant TCH4 is an XET that is specific for xyloglucans; it is able to cleave both fucosylated and nonfucosylated xyloglucans as donor substrates. The preferred acceptor substrate appears to be the nonreducing terminus of high- M_r xyloglucan, but TCH4 will also utilize xyloglucan-derived oligosaccharides. Oligosaccharides with two galactosyl side chains are preferred over oligosaccharides with a fucosyl-galactosyl side chain. The optimum pH of the enzyme is 6.0 to 6.5, inconsistent with a role in acid-induced cell wall loosening. The TCH4 temperature optimum of 12 to 18°C and high activity at 0°C suggest that

TCH4 may play a role in cold acclimation and/or cold-tolerant growth. TCH4 activity is enhanced by urea and BSA, but not cations, reducing agents, or carboxymethylcellulose. The detailed characterization of the other members of the XET-related protein family will help to elucidate how these different proteins affect the cell wall during development and in response to hormonal and environmental stimuli.

APPENDIX

The following analysis, which assumes that donor and acceptor substrates bind to the enzyme independently of each other, provides an estimate of the affinity of the acceptor site for xyloglucan.

The hypothetical linear Lineweaver-Burk plot, which would be expected if xyloglucan did not occupy the enzyme's acceptor site, is given by:

$$1/v = (1/V_{\max(d)}) \times \{1 + (K_{md}/[S_d])\} \quad (1)$$

where v is the rate of [^3H]XLLGol incorporation, $V_{\max(d)}$ is the theoretical rate at infinite donor (xyloglucan) concentration and an arbitrarily chosen [^3H]XLLGol concentration, $[S_d]$ is the concentration of xyloglucan as the donor substrate, and K_{md} is the Michaelis constant for xyloglucan as the donor substrate ≈ 0.62 mg/mL (see Fig. 4B).

The rate of ^3H incorporation, v , is also given by:

$$1/v = (1/V_{\max(a)}) \times \{1 + (K_{ma}/[S_a])\} \quad (2)$$

where $V_{\max(a)}$ is the theoretical rate at the infinite acceptor [^3H]XLLGol concentration and an arbitrarily chosen xyloglucan concentration, $[S_a]$ is the concentration of [^3H]XLLGol as the acceptor substrate; and K_{ma} is the Michaelis constant for [^3H]XLLGol as the acceptor substrate = 73 μM .

Thus, under the conditions of our experiment, where $[S_a] = 8 \mu\text{M}$, it follows that $v/V_{\max} = 0.09877$. This implies that 9.877% of the enzyme molecules would have their acceptor sites occupied by [^3H]XLLGol at any moment if xyloglucan did not occupy the acceptor site.

However, xyloglucan also appears to occupy the enzyme's acceptor site, acting as a competitive inhibitor of [^3H]XLLGol incorporation; the inhibited rate of ^3H incorporation is thus given by:

$$1/v = 1/V_{\max(a)} + \{K_{ma}(1 + [I]/K_i)/V_{\max}[S_a]\} \quad (3)$$

where $[I]$ is the concentration of inhibitor and K_i is the dissociation constant of the enzyme-inhibitor complex (i.e. the K_m for xyloglucan at the acceptor site).

Since $K_{ma} = 73 \mu\text{M}$ and $[S_a] = 8 \mu\text{M}$, Equation 3 gives:

$$v/V_{\max(a)} = \{1 + 9.125(1 + [I]/K_i)\}^{-1} \quad (4)$$

In the special case where $[I] = K_i$, it follows that $v/V_{\max} = 0.05195$, which means that the effective concentration of enzyme, $[E']$, is 0.05195/0.09877, or 52.6% of what it would have been if xyloglucan had not acted as a competitive inhibitor. In general, $[E']$ is given by:

$$[E'] = 1/0.09877\{1 + 9.125(1 + [I]/K_i)\} \quad (5)$$

Thus, when xyloglucan competitively displaces [^3H]X-LLGol from the acceptor site, Equation 1 requires a revised $V_{\max(d)}$ termed $V'_{\max(d)}$ where

$$V'_{\max(d)} = [E'] \times V_{\max(d)} \quad (6)$$

Using $V'_{\max(d)}$ in place of $V_{\max(d)}$ inserting the value $K_{\text{md}} = 0.62 \text{ mg/mL}$, and noting that $[I] = [S_d]$, Equation 1 becomes:

$$1/v = (0.09877\{1 + 9.125(1 + [S_d]/K_i)\}/V_{\max(d)}) \times \{1 + (0.62/[S_d])\} \quad (7)$$

The data of Figure 4C, left, are shown as a double-reciprocal plot in Figure 4C, right. We used the Marquardt-Levenberg algorithm (available on SigmaPlot, Jandel Scientific Software, Erkrath, Germany) to find, by iteration, the values of $V_{\max(d)}$ and K_i that give the best fit to the experimental data points. These values are $V_{\max(d)} = 64 \pm 7 \text{ Bq kBq}^{-1} \text{ h}^{-1} \mu\text{g}^{-1}$ and $K_i = 0.26 \pm 0.05 \text{ mg/mL}$. The smooth curve in Figure 4C, right, is calculated from these constants using Equation 7, and shows an acceptable fit to the data. The dashed line (AG) shows the hypothetical linear plot that would have been expected (Eq. 1, with $V_{\max(d)} = 64 \text{ Bq kBq}^{-1} \text{ h}^{-1} \mu\text{g}^{-1}$) if xyloglucan had not occupied the acceptor site: intersect A is $-1/K_{\text{md}}$, and intersect B is $1/V_{\max(d)}$. Curves AG and CF converge at ∞ . The value of $1/[\text{xyloglucan}]$ where the theoretical $1/v$ is 52.6% of the observed $1/v$ (i.e. where DE/CE = 0.526) is an estimate of $1/K_i$. We interpret this as showing that the K_m of the enzyme for xyloglucan as the acceptor substrate is approximately 0.26 mg/mL , which equals approximately $0.3 \mu\text{M}$ (to one significant figure).

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